

# Identification of Neutrophil Elastase as the Proteinase in Burn Wound Fluid Responsible for Degradation of Fibronectin

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To identify proteinases responsible for fibronectin degradation in the wound environment we studied wound fluid obtained from burn patients. Immunoblotting experiments showed that extensive degradation of fibronectin had occurred in some burn wound fluid samples, in which case intact fibronectin molecules were undetectable, and the largest fibronectin fragment was 116 kDa. The 116-kDa fragment as well as a smaller 90-kDa fragment contained the fibronectin cell binding domain. These burn-fluid samples degraded freshly added fibronectin. Activity of the fibronectin-degrading enzyme was blocked by a broad-spectrum serine proteinase inhibitor or by specific neutrophil elastase inhibitors but not by metalloproteinase inhibitors or inhibitors

of trypsin-like or chymotrypsin-like serine proteinases. Enzyme activity also was neutralized by antibodies against human neutrophil elastase. Incubation of fibronectin with burn wound fluid or purified human neutrophil elastase generated similar fibronectin-degradation products. Finally, direct assay of burn-wound-fluid samples with a synthetic elastase substrate showed a correlation between fluid-phase elastase activity and fibronectin degradation. Based on these findings, we conclude that burn-wound-fluid elastase is responsible for extensive fibronectin degradation. Acute elevation of elastase did not appear to hinder normal wound repair.

**Key words:** wound healing/metalloproteinase/serine proteinase. *J Invest Dermatol* 103:155–161, 1994

**N**ormal wound healing requires precise temporal and spatial regulation of cell adhesion proteins, proteinases, and growth factors, and inappropriate expression of any of these factors could result in abnormal healing [1,2]. In our research we have focused on the cell adhesion protein fibronectin and the various roles that fibronectin plays in wound repair [3,4]. Fibronectin is deposited at the wound interface at the time of initial wounding and subsequently becomes part of the provisional matrix [5,6].

Recently, we began experiments aimed at characterizing fibronectin and its possible proteolysis in the wound environment. We found that wound fluid from chronic venous stasis ulcers contains degraded fibronectin [7,8] and activated metalloproteinases [9]. Because fibronectin is important for keratinocyte migration and wound re-epithelization [10], persistent degradation of fibronectin and other extracellular matrix components could be a factor in failed wound closure [11].

To learn whether fibronectin degradation occurs in acute traumatic wounds, we studied wound fluid obtained from burn patients. Plasma fibronectin decreases markedly after burn injury [12], and some fibronectin fragments can be detected in the blood [13]. Previously, we found that gelsolin degradation occurred in some burn-wound-fluid samples [14]. Therefore, we anticipated that fibronectin degradation also might occur in burn wounds. Our studies

show that burn-wound fluid contains fibronectin fragments, and neutrophil elastase has been identified as the enzyme responsible for extensive fibronectin degradation.

## MATERIALS AND METHODS

**Wound Fluid and Plasma** This research project was approved by the University Institutional Review Board. Informed consent was obtained for all procedures. Burn-wound-fluid samples were collected from patients admitted to the burn unit of a large metropolitan hospital. Patients had sustained partial to full-thickness burns involving at least one extremity. Table I summarizes the patient population used in the study.

Wound-fluid samples were collected immediately following routine cleansing of the burn wounds with 4 × 4 sterile gauze and Hibiclen soap (Stuart Pharmaceuticals). This procedure, which is routinely carried out with patients 1–2 times/d, removes the fibrous exudate that forms over their burn wounds. A sterile glove (gas autoclaved, Bodi-gard shoulder length, polyethylene glove, Hospital Therapy Products, Inc.) was applied to the extremity and secured at its proximal aspect with a noncompressive cling wrap. This was left in place 4 h, during which time burn-wound fluid accumulated. Subsequently, the burn fluid (5–10 ml) was drained from the glove. Some samples of burn fluid were sent to hematology for differential white blood cell counts, but numerical results were not possible because few intact cells were observed.

Wound-fluid samples were centrifuged at 13,500 rpm (Beckman J2-21M, 20 rotor) for 15 min at 22°C. Supernatants were frozen in liquid N<sub>2</sub> and stored at –70°C until use. Blood samples were obtained by venipuncture with a 21-gauge stainless steel needle attached to a blood collection device (Vacutainer, Becton Dickinson, Rutherford, NJ) containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. To obtain plasma, cells were removed from the samples by centrifugation (1800 × g for 15 min at 22°C followed by 22,000 × g for 30 min at 4°C). The supernatants were filter sterilized using a 0.2-μm filter, and stored at –70°C until use. Protein concentrations were determined by the Lowry method [15].

**SDS-PAGE and Immunoblotting** Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and cell blotting

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Abbreviations: AEBSF, aminoethylbenzenesulfonylfluoride; BHK, baby hamster kidney; DPBS, Dulbecco's phosphate-buffered saline; MMP, matrix metalloproteinase.

Table I. Burn Patient Profile<sup>a</sup>

Number	Age	Burn Size and Depth	Sampling Day
1	21	15%—partial	1
2	29	15%—partial	5
3	28	16%—partial	1
4	66	30%—5% full	1
5	77	20%—5% full	2
6	48	77%—partial	8
7	41	83%—42% deep	8
8	32	15%—partial	2
9	51	30%—14% full	6
10	35	8%—partial	3
11	70	32%—30% full	5

<sup>a</sup> See Materials and Methods for details.

were carried out as described previously [8]. Briefly, samples for SDS-PAGE [16] were dissolved in reducing sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH 6.8, 5% mercaptoethanol) unless indicated otherwise and subjected to electrophoresis on 7.5% acrylamide mini-gels (Bio-Rad Mini-Protein apparatus) at 22°C for 45 min and 200 V. Bio-Rad high molecular mass standards were used for markers.

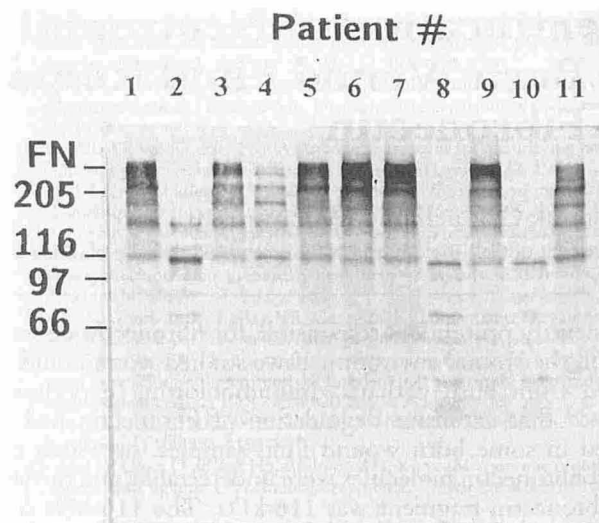
For immunoblotting, polypeptides separated by SDS-PAGE were transferred to nitrocellulose paper (Schleicher & Schuell) by electrophoresis at 22°C for 1 h at 100 V or at 22°C for 16 h at 34 V. The transferred proteins were incubated with polyclonal anti-fibronectin antibodies for 2 h at 22°C followed by incubation with alkaline-phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) for 1 h at 22°C. Visualization was accomplished using the Bio-Rad alkaline-phosphatase conjugate substrate kit according to the manufacturer's instructions.

For cell blotting, polypeptides separated by SDS-PAGE were transferred to nitrocellulose paper as described above. The transferred protein strips were blocked with 3% bovine serum albumin (BSA) (Miles, Fraction V) in Tris-buffered saline (TBS) (20 mM Tris base, 0.5 M NaCl, pH 7.5), then washed with TTBS (TBS + 0.05% Tween 20) followed by Dulbecco's phosphate buffered saline (DPBS) (150 mM NaCl, 3 mM KCl, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 6 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2). Baby hamster kidney (BHK) was cultured overnight with 20  $\mu$ Ci/ml of <sup>35</sup>S-methionine (ICN, 1100 Ci/mmol) in methionine-deficient DME medium (GIBCO), harvested, and cultured 3–4 h in suspension in fresh medium without radiolabeled methionine. Each nitrocellulose strip was incubated for 1 h at 37°C with 10<sup>6</sup> cells (3.25  $\times$  10<sup>6</sup> cpm) in 0.5 ml DPBS containing 30 mg/ml bovine serum albumin (Miles, Fraction V). At the end of the incubations, unattached cells were removed by washing three times with TTBS. The attached cells were fixed with 3% paraformaldehyde and dried for autoradiography. Radioactive bands show the locations of adhesion-promoting polypeptides.

**Zymography** Proteinase profiles were determined as described previously [9] by zymography [17]. Briefly, samples of burn-wound fluid were subjected to SDS-PAGE on gelatin-containing acrylamide gels (7.5% acrylamide and 0.5% gelatin) under non-reducing conditions. After electrophoresis, gels were washed twice with 2.5% Triton X100 for 30 min to remove SDS. They were then rinsed briefly with H<sub>2</sub>O followed by incubation overnight at 37°C in reaction buffer containing 50 mM Tris, 150 mM NaCl, and 5 mM CaCl<sub>2</sub> (pH 7.4). At the end of the incubations, the gels were stained with 0.125% Coomassie brilliant blue. Areas of proteinase activity appeared as clear zones against a dark blue background, and this method detects metalloproteinases even if they are still in the proenzyme form [17].

**Elastase Determination** Burn-wound fluid or plasma samples (25–50  $\mu$ g) or human neutrophil elastase (0.02–0.2 million units) were incubated 1 h at 22°C in 1 ml of 0.1 M Hepes buffer, pH 7.5, containing 0.5 M NaCl, 10% dimethylsulfoxide, and 0.1 mM elastase substrate (methoxysuccinyl-alanyl-alanyl-p-nitroanilide) [18]. Substrate degradation was determined by measuring OD<sub>410</sub> (Beckman DU-40 spectrophotometer). A standard curve for degradation was prepared from the elastase data, which ranged from 0 to 0.44 OD units. Burn fluid and plasma results were converted to million units (mU) of elastase activity and normalized to mg burn-fluid protein.

**Materials** Human plasma fibronectin was obtained from the New York Blood Center. BHK cells were cultured as described previously [8]. Aminocaprylsulfonate (AEBSF), human neutrophil elastase (22 units/mg), human neutrophil cathepsin G (2 units/mg), elastase substrate, and elastinal were purchased from Calbiochem Co. Aprotinin, chymosta-



**Figure 1.** Fibronectin profiles in burn-wound fluid. Samples (30  $\mu$ g/lane) of burn-wound fluid from the patients indicated were subjected to SDS-PAGE and analyzed by immunoblotting with anti-fibronectin. Other details are in Materials and Methods.

tin, leupeptin, EDTA, 1,10-phenanthroline, gelatin (bloom 225), casein, and plasmin (4.2 units/mg) were purchased from Sigma Co. Trypsin (1:250) was obtained from GIBCO. The elastase inhibitor ICI 200,355 was a gift from ICI Americas Inc., and the elastase inhibitor MDL 27,367 was a gift from Marion Merrell Dow Research Inst. Goat anti-human neutrophil elastase (purified IgG, 18 mg/ml) was purchased from ICN Biomedicals, Inc. Monoclonal antibody 7-11C against MMP-9 (92-kDa matrix metalloproteinase) was a gift from Dr. Debbie French, SUNY Stonybrook.

## RESULTS

### Fibronectin Profiles in Burn-Wound Fluid and Plasma

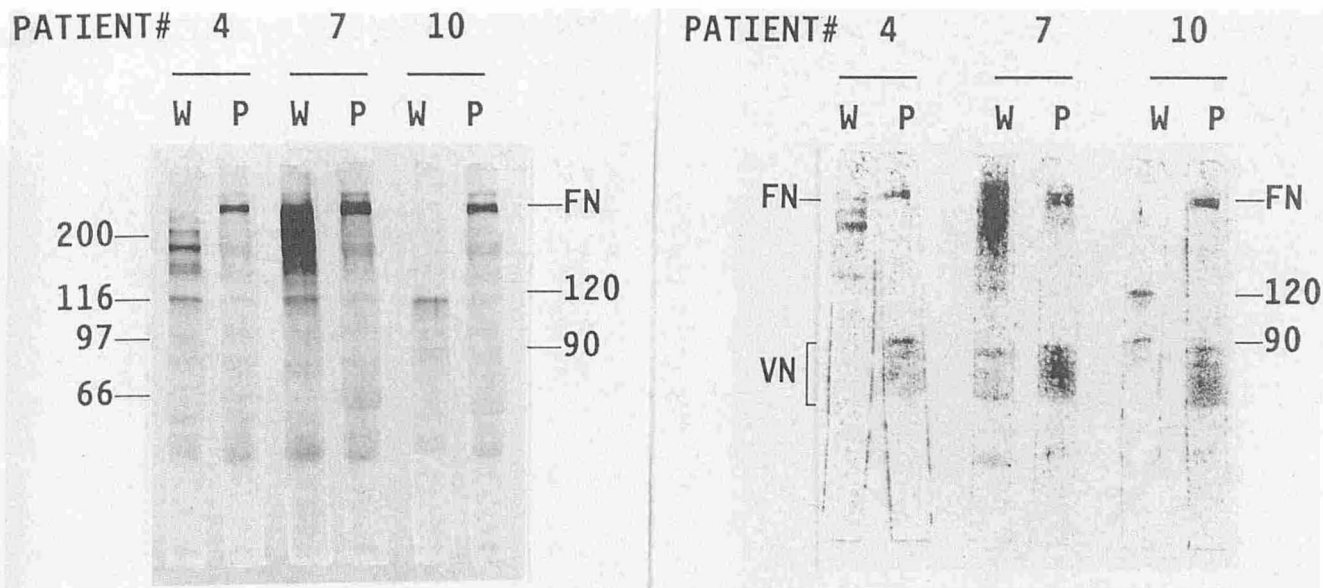
Figure 1 shows immunoblotting profiles of fibronectin in burn-wound fluid samples from 11 patients. Degradation of fibronectin was observed in every sample. With patients 1, 3–7, 9, and 11, the samples contained several bands ranging from 120 kDa to intact fibronectin. With patients 2, 8, and 10, no intact fibronectin remained, and the major fragment was 116 kDa.

Figure 2 shows typical results comparing fibronectin profiles (left) and cell-blotting profiles (right) of burn fluid and plasma from several of the patients. Cell blotting identifies those fragments that contain cell adhesion recognition domains [8,19]. Plasma samples from all patients contained mostly intact fibronectin and vitronectin (65–75 kDa).

In wound fluid with partially degraded fibronectin, the 120-kDa and higher-molecular-mass fragments observed by immunoblotting also could be detected by cell blotting, suggesting that these fragments contained the fibronectin cell binding domain. In samples with extensively degraded fibronectin, the 116-kDa fibronectin fragment and a smaller 90-kDa fragment also had cell-binding activity. Cell-blotting studies also indicated that vitronectin was reduced or absent in wound fluid with partially degraded fibronectin and absent from wound fluid with extensively degraded fibronectin.

**Proteinases in Burn-Wound Fluid** In addition to adhesion protein fragments, all burn-wound-fluid samples contained proteinases that could be detected by gelatin zymography [17]. The highest activity was found in samples 2, 8, and 10 [14], which showed the most extensive fibronectin degradation. Figure 3 shows results for wound fluid sample 10 using trypsin (TR) as a control. In the wound fluid, there were 6–7 prominent gelatinase bands ranging from 66 kDa to more than 230 kDa. In corresponding plasma samples, gelatinase activity was barely detectable (data not shown).

The band migrating at about 97 kDa was identified as proenzyme



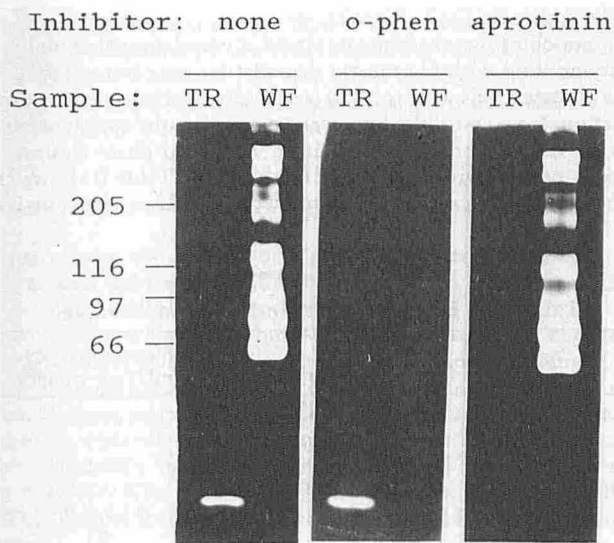
**Figure 2.** Immunoblotting and cell-blotting analysis of fibronectin fragments. Samples (30  $\mu$ g/lane) of burn-wound fluid (W) and plasma (P) from patients 4, 7, and 10 were subjected to SDS-PAGE and analyzed by immunoblotting (left) and cell blotting (right). Positions of fibronectin (FN) and vitronectin (VN) are determined with standards. Other details are in *Materials and Methods*.

MMP-9 (94-kDa type IV collagenase) by immunoblotting with monoclonal antibody 7-11C, and the band at about 66 kDa co-migrated with mastectomy fluid MMP-2 (72-kDa type IV collagenase) (data not shown). Figure 3 also shows that all of the gelatinase bands were metalloproteinases because they could be inhibited by o-phenanthroline (o-phen), which had no effect on trypsin activity. In contrast, trypsin was inhibited by aprotinin, which had no effect on wound fluid gelatinases.

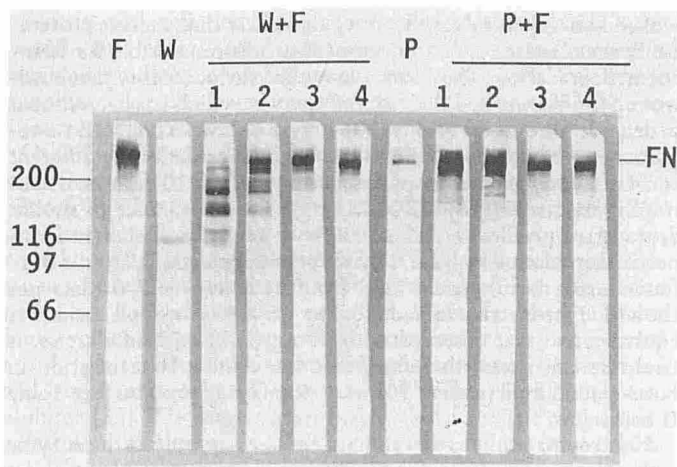
The results in Fig 3 indicated that, compared to plasma, burn-wound fluid contains high levels of several metalloproteinases. Because zymographic analysis detects proteinases regardless of whether they are in the inactive proenzyme or activated enzyme

form [17], direct proteolysis experiments also were carried out. Figure 4 shows an experiment in which different concentrations of wound fluid or plasma were incubated with fibronectin, after which the extent of fibronectin degradation was determined by immunoblotting. Substantial fibronectin degradation occurred after incubation with wound fluid (lane 1, 30  $\mu$ g; lane 2, 3  $\mu$ g) but not plasma.

**Identification of Elastase as the Proteinase in Wound Fluid Responsible for Fibronectin Degradation** Using the fibronectin proteolysis assay, we analyzed the type of proteinase involved in fibronectin degradation. Figure 5 shows that proteolysis of fibronectin could be blocked by the broad spectrum serine proteinase inhibitor AEBSF (lane 3 compared to lane 2). On the other hand, proteolysis was not blocked by addition of the metalloproteinase

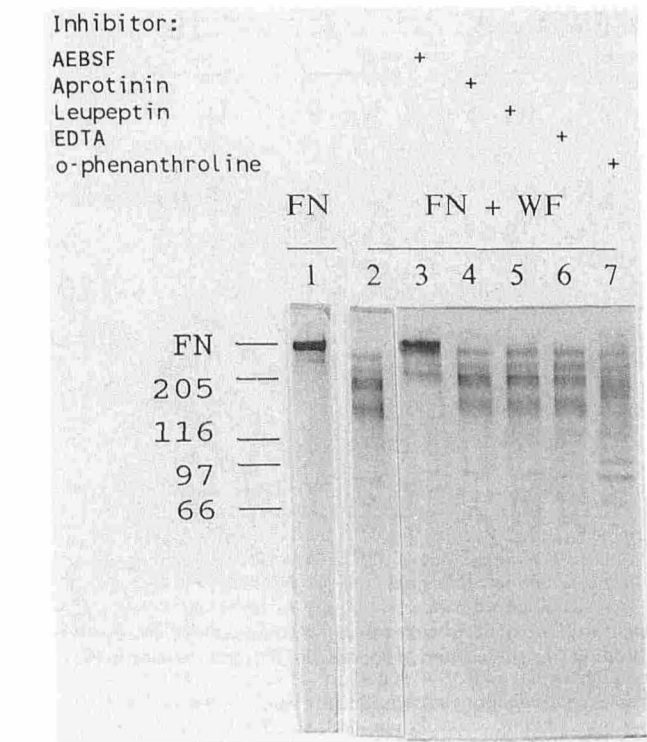


**Figure 3.** Gelatinase zymography of burn-wound fluid. Samples of trypsin (TR) (0.2  $\mu$ g/lane) and patient 10 burn-wound fluid (WF) (5  $\mu$ g/lane) were subjected to gelatin zymography. O-phenanthroline (10 mM) (a metalloproteinase inhibitor) and aprotinin (20  $\mu$ g/ml) (a trypsin/serine proteinase inhibitor) were added to the reaction buffer where indicated. Wound-fluid samples from patients 2 and 8 showed identical gelatinase profiles (not shown). Other details are in *Materials and Methods*.



**Figure 4.** Degradation of fibronectin by burn-wound fluid. Samples of fibronectin (F) (0.22  $\mu$ g) were mixed with patient 10 burn-wound fluid (W) or plasma (P) in 14  $\mu$ l DPBS. After 5 min at 37°C the reactions were terminated by the addition of SDS-sample buffer, and the samples were subjected to SDS-PAGE and immunoblotting with anti-fibronectin. The amounts of burn-wound fluid and plasma were 30  $\mu$ g (lane 1), 3  $\mu$ g (lane 2), 0.3  $\mu$ g (lane 3), and 0.03  $\mu$ g (lane 4). Other details are in *Materials and Methods*.



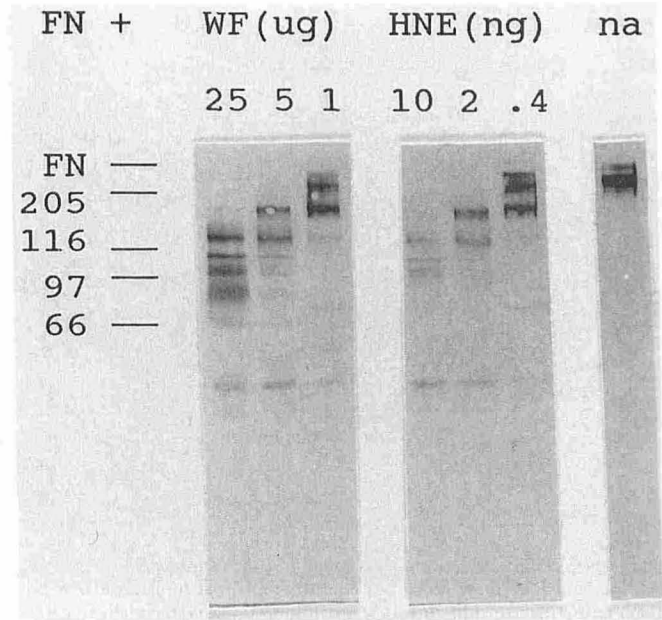


**Figure 5.** Effects of proteinase inhibitors on fibronectin degradation by burn-wound fluid. Burn-wound fluid (patient 10, 5  $\mu$ g) in 12  $\mu$ l DPBS was preincubated for 10 min at 37°C with AEBSF (1.7 mM), aprotinin (17  $\mu$ g/ml), leupeptin (0.8 mM), EDTA (8 mM), and o-phenanthroline (8 mM) as indicated. Subsequently, fibronectin (0.5  $\mu$ g) in 4  $\mu$ l phosphate saline buffer (10 mM  $\text{NaH}_2\text{PO}_4$ , 150 mM NaCl, pH 7.2) was added to the incubations, which were allowed to continue for an additional 60 min. The reactions were terminated by the addition of SDS-sample buffer, and the samples were subjected to SDS-PAGE and immunoblotting with anti-fibronectin. Other details are in *Materials and Methods*.

inhibitors o-phenanthroline and EDTA or by the addition of the serine proteinase inhibitors aprotinin and leupeptin. The latter block the trypsin class of serine proteinases but not the chymotrypsin or elastase-related enzymes.

Based on the above results, it seemed likely that a serine proteinase in the elastase or chymotrypsin class was responsible for fibronectin degradation. Therefore, we studied the possibility that burn-wound fluid contained the enzyme elastase, which has been shown to degrade fibronectin *in vitro* [20]. Figure 6 shows a typical dose-response experiment comparing fibronectin degradation by different concentrations of burn-wound fluid from patient 10 or human neutrophil elastase (HNE) (22 units/mg). The appearance of similar degradation profiles was consistent with a role for elastase in fibronectin degradation by burn fluid. Similar degradation patterns were found using burn-wound fluid from patients 2 and 8 (data not shown). Fibronectin degradation by 25  $\mu$ g of wound fluid was equivalent to that observed with 10 ng (0.22 mU) of elastase. It could be calculated, therefore, that the elastase concentration in burn-wound fluid (patient 10) was  $\sim$ 9 mU/mg protein. (See Table II below.)

Neutrophil granules that contain elastase also contain the enzyme cathepsin G, a chymotrypsin-related serine proteinase that can degrade fibronectin [21]. Therefore, we checked whether wound-fluid cathepsin G played a role in fibronectin degradation. Figure 7 shows an experiment comparing fibronectin degradation by elastase, cathepsin G, and wound fluid in the presence of specific inhibitors of neutrophil elastase, ICI 200,355 [22], and MDL 27,367 [23], or in the presence of chymostatin, an inhibitor of chymotrypsin-related serine proteinase. The ICI and MDL compounds inhibited



**Figure 6.** Comparison of fibronectin degradation by burn-wound fluid and human neutrophil elastase. Samples of fibronectin (0.5  $\mu$ g) were mixed with patient 10 burn wound fluid (WF) or human neutrophil elastase (HNE) at the concentrations indicated in 22  $\mu$ l DPBS. After 60 min at 37°C the reactions were terminated by the addition of SDS-sample buffer, and the samples were subjected to SDS-PAGE and immunoblotting with anti-fibronectin. Other details are in *Materials and Methods*.

fibronectin degradation by elastase but not by cathepsin G, whereas chymostatin inhibited fibronectin degradation by cathepsin G but not by elastase. In related experiments, samples of wound fluid (patient 10), elastase, and cathepsin G were incubated with polyclonal antibodies against human neutrophil elastase and then tested for fibronectin degradation. Addition of antibody (9  $\mu$ g IgG) completely neutralized fibronectin degradation by 4 ng neutrophil elastase or 5  $\mu$ g wound fluid but had no effect on fibronectin degradation by cathepsin G (2–12 ng).

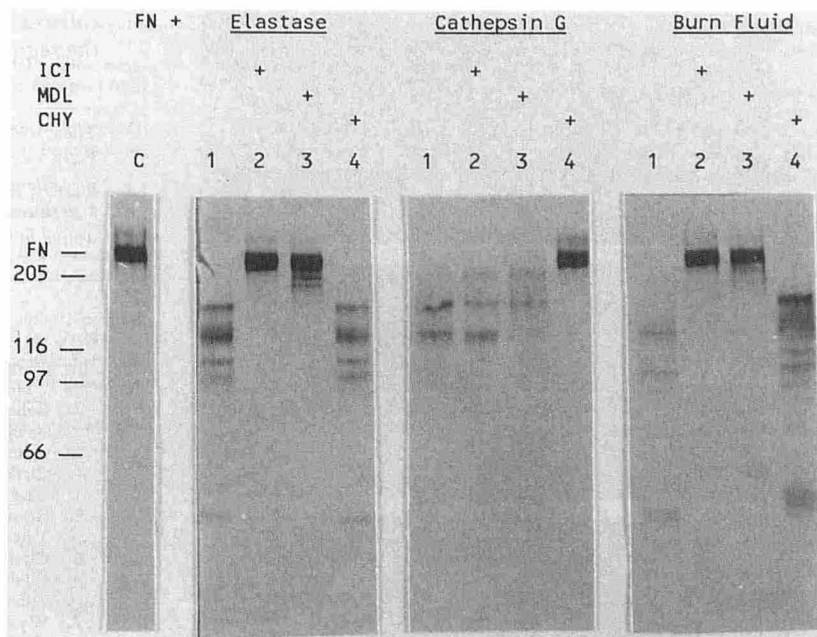
Immunoblotting experiments aimed at correlating elastase levels in wound fluid with fibronectin degradation were not possible because elastase levels were too low (relative to total protein) to permit detection. It was possible, however, to correlate the extent of burn-wound–fluid fibronectin degradation with fluid-phase elastase activity using a spectrophotometric method [18]. Table II shows that high elastase levels occurred in burn-wound fluids with extensively

**Table II.** Elastase Activity in Burn-Fluid Samples<sup>a</sup>

Experiment	Sample	Elastase Activity (mU/mg sample)
1	10-BF	14.6 $\pm$ 1
	10-PL	<1
2	2-BF	7.2 $\pm$ 0.4
	8-BF	6.0 $\pm$ 0.4
	10-BF	14.5 $\pm$ 0.4
	6-BF	<1
	7-BF	1.6 $\pm$ 1.6
	9-BF	<1

<sup>a</sup> Samples of burn-wound fluid (BF) or plasma (PL) from the patients indicated were incubated with elastase substrate. Substrate degradation was determined by measuring OD<sub>410</sub>. The results were converted to milliunits (mU) of elastase based on a standard curve and normalized to protein concentration. Data shown are averages  $\pm$  SD from duplicate determinations. Other details are in *Materials and Methods*.

**Figure 7.** Effect of elastase inhibitors on fibronectin degradation by burn-wound fluid, human neutrophil elastase, and human cathepsin G. Samples of elastase (2 ng), cathepsin G (2 ng), or patient 10 burn fluid (5  $\mu$ g) as indicated were preincubated with ICI 200,355 (10  $\mu$ M), MDL 27,367 (20  $\mu$ M), or chymostatin (200  $\mu$ M) in 17  $\mu$ l DPBS. After 10 min at 37°C, fibronectin (0.5  $\mu$ g) was added to the incubations, which were allowed to continue for an additional 60 min. The reactions were terminated by the addition of SDS-sample buffer, and the samples were subjected to SDS-PAGE and immunoblotting with anti-fibronectin. Other details are in *Materials and Methods*.



degraded fibronectin (patients 2, 8, and 10) whereas low or undetectable elastase activity occurred in burn-wound fluids with minimally degraded fibronectin (patients 6, 7, and 9). (See Fig 1.) No elastase activity was detectable in 25–50- $\mu$ g samples of plasma. The level of burn wound fluid elastase measured by the spectrophotometric assay (6–15 mU/mg) corresponded well with the level of elastase measured by the fibronectin degradation assay ( $\sim$ 9 mU/mg) (Fig 6).

**Effect of Burn Wound Fluid on Cell Spreading** Taken together, the results described above show that elastase was the proteinase in burn fluid responsible for degradation of fibronectin. Because fibronectin plays an important role in cell adhesion during wound repair [3,4] we tested whether burn-wound fluid elastase could inhibit cell adhesion. BHK fibroblasts were allowed to attach and spread on fibronectin-coated culture dishes (Fig 8A). Figure 8B shows that the addition of burn fluid to the previously spread BHK cells caused cell rounding, and that this activity could be neutralized by the elastase inhibitors ICI 200,355 (Fig 8C) or MDL 27,367 (data not shown). These data indicate that burn fluid elastase can interfere with cell adhesion to fibronectin.

## DISCUSSION

To learn more about the wound-healing process, we have been using wound fluid as a non-invasive method to characterize adhesion proteins and proteinases in the wound environment. In the present studies, we analyzed burn-wound fluid to learn if fibronectin degradation occurred in acute traumatic wounds. Immunoblotting experiments showed that burn fluid contained degraded fibronectin. In some samples, degradation resulted in a mixture of intact fibronectin molecules and fragments 120 kDa and larger. In others, intact fibronectin molecules were absent, and the largest fragment was 116 kDa. The 116-kDa fragment as well as a smaller 90-kDa fragment both contained the fibroblast cell binding domain.

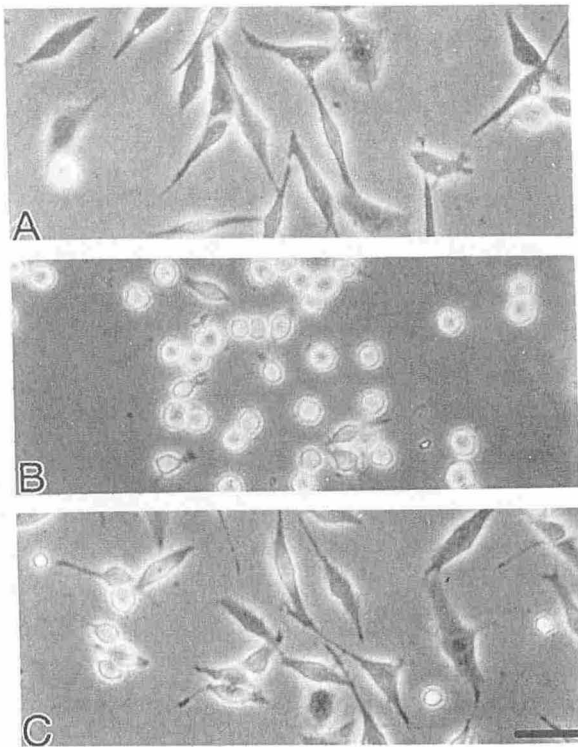
Wound-fluid samples containing extensively degraded fibronectin contained a proteinase capable of degrading freshly added fibronectin. Activity of the enzyme was blocked by a broad spectrum serine proteinase inhibitor and by specific inhibitors of neutrophil elastase but not by metalloproteinase inhibitors or inhibitors of trypsin-like or chymotrypsin-like serine proteinases. Moreover,

wound-fluid fibronectin-degradation activity was neutralized by antibodies against human neutrophil elastase. Incubation of fibronectin with burn-wound fluid or purified human neutrophil elastase generated similar fibronectin degradation products, and direct assay of burn-wound fluid samples with a synthetic elastase substrate showed a correlation between elastase activity and fibronectin degradation. These findings suggest that neutrophil elastase is the fibronectin-degrading proteinase in wound fluid. In addition to elastase, neutrophils contain an elastase-like enzyme called proteinase 3, whose broad range of substrates includes fibronectin [24,25]. Our results cannot exclude the possibility that proteinase 3 also plays a role in fibronectin degradation in burn-wound fluid.

A role for burn-fluid elastase in fibronectin degradation is consistent with other findings, e.g., the release of elastase from neutrophils, which occurs as part of the inflammatory response [26,27], and reported ability of elastase to degrade fibronectin *in vitro* [20]. Moreover, adding activated neutrophils to cell cultures *in vitro* results in elastase-dependent degradation of extracellular matrix fibronectin and cell detachment [28–30]. In our studies, burn-wound-fluid elastase inhibited fibroblast adhesion to fibronectin. Finally, degradation of fibronectin by elastase has been described in bronchial secretions from patients with cystic fibrosis [31] and emphysema [32].

Other investigators reported that elevated elastase levels occur in patient serum 24 h following burn injury that are greater than 40% TBSA [33]. Using a similar assay method, but approximately 100-times smaller samples, we detected elastase activity in patient wound fluid but not in plasma. It seems likely, therefore, that elastase in wound fluid is released locally by neutrophils in the wound matrix, not derived from the circulation. Moreover, there are sufficient levels of  $\alpha$ 1-proteinase inhibitor to neutralize elastase released into the plasma after burn injury [34], whereas some burn-wound fluid samples were found to have markedly reduced levels of elastase inhibitor [35]. The presence of active elastase at the wound site indicates an imbalance between the proteinase and its serum inhibitors. We have yet to determine whether this imbalance occurs because local elastase release by neutrophils sometimes exceeds wound proteinase inhibitor capacity or because there are decreased levels of proteinase inhibitors at the wound site.

Previous studies on chronic wound fluid had led us to anticipate that gelatinases might play a role in fibronectin degradation [9,14]. The present results suggest that although MMP-9 and other gelatinases are elevated in wound fluid samples containing degraded



**Figure 8.** Reversal of cell spreading by wound fluid and the effect of elastase inhibitors. Culture dishes were coated with fibronectin (10  $\mu$ g/ml in DPBS) and then incubated 2 h with BHK cells in DMEM medium (GIBCO) and 10% fetal bovine serum during which time cell spreading occurred. Subsequently, the incubations were continued for an additional 90 min in DMEM containing (A) 10% serum; (B) 2 mg/ml burn-wound fluid 10; or (C) 2 mg/ml burn-wound fluid 10 with 10  $\mu$ M ICI 200,355. The final DMSO concentration in all samples was 2%. At the end of the incubations, samples were photographed using a Zeiss inverted microscope. Bar, 40  $\mu$ M.

fibronectin, these enzymes probably are not responsible for fibronectin degradation. The observation that both elastase and MMP-9 are increased in wound fluid is consistent with neutrophils as the source for elastase and cathepsin G [26] as well as MMP-9 [36,37]. In burn-fluid samples with partially degraded fibronectin, the proteinase responsible for fibronectin degradation has yet to be identified. Based on differences in the fibronectin degradation profiles, it appears that different enzymes or combinations of enzymes result in partial and extensive degradation of fibronectin. Therefore, gelatinases or other metalloproteinases might be involved.

Five of the 11 patients (1, 2, 3, 8, and 10) were discharged from the burn unit within 2 weeks after injury without requiring surgical intervention. Of these, three had extensively degraded fibronectin on the day their burn-wound fluid was sampled. Therefore, degradation of fibronectin, even extensive degradation, is not by itself an indicator of non-healing conditions. Transient fibronectin degradation might even be beneficial because some fibronectin fragments have unique biologic activities that could promote repair, e.g., through chemoattraction of monocytes [38,39] or by increasing monocyte phagocytic activity [40], neutrophil adhesiveness [41], and fibroblast proliferation [42].

Although acute elevation of elastase did not appear to hinder wound repair, chronically high levels of elastase would likely have pathophysiologic consequences including degradation of the dermal/epidermal junction [43]. As shown in this study, burn-fluid elastase can interfere with fibroblast adhesion to fibronectin. Persistent elastase-mediated degradation of extracellular matrix components in the wound bed could account for leukocyte-mediated chronic wound damage [44] and might play a role in development of chronic skin ulcers [11]. In future studies using longitudinally

collected burn wound fluid samples, we hope to learn more about the regulation of elastase and other proteinases in wound fluid.

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